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AUTHORITY

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Award Number: DAMD17-96-1-6306

TITLE: Genetic and Dynamic Analyses of Murine Peak Bone Density

PRINCIPAL INVESTIGATOR: Wesley G. Beamer, Ph.D.

CONTRACTING ORGANIZATION: Jackson Laboratory
Bar Harbor, Maine 04609-1500

REPORT DATE: October 1999

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
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Patricia O'Modem 5/8/97

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13. ABSTRACT *(Maximum 200 words)*

During our third year, the major effort has continued with genetic analyses of normal bone mineral density using inbred strains of mice differing by 35-50% in volumetric BMD. All (C57BL/6 x C3H/He)F2 and (C57BL/6 x CAST/Ei)F2 mice have been phenotyped for femoral vBMD and (B6 x C3H)F2 have been phenotyped for vertebral vBMD. Both F2 populations have been genotyped for the 10% of mice with the highest and 10% with the lowest bone densities. Genome-wide analyses of (B6 x CAST)F2 mice revealed strong vBMD loci on 4 Chromosomes (Chrs 1, 5, 13, 15) and suggestive vBMD loci on 4 additional Chrs (3, 4, 10, & 14). Genome wide analyses of (B6 x C3H)F2 mice revealed strong vBMD loci on 5 Chrs (1, 4, 11, 13, & 18) and suggestive vBMD loci on 3 additional Chrs (6, 14, & 16). Congenic strains carrying Chr regions from CAST (1, 3, 5, 13, & 14) or regions from C3H (1, 4, 6, 13, &18) have significantly altered femoral vBMD as a consequence of the donated Chr segment. Preliminary mapping data for (B6 x C3H)F2 vertebrae reveal 4 vBMD loci shared with femurs (1, 4, 14, & 18) and 2 unique loci (Chr 7 & 9). Lastly, a new DEXA instrument for mice, the PIXImus, has been validated for body composition, and demonstrated capable of detecting the major loci for BMD shared with the femoral bone site.

14. SUBJECT TERMS
Osteoporosis, Genetics, Bone Mineral Density

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

W. D. Beamer 10/27/99
PI - Signature Date

TABLE OF CONTENTS

LOMA LINDA UNIVERSITY

School of Medicine
Department of Medicine

Loma Linda, California 92350

LETTER OF INTENT TO ENTER INTO A CONSORTIUM AGREEMENT

TITLE OF APPLICATION:	Genetic and Dynamic Analyses of Murine Peak Bone Density
APPLICANT ORGANIZATION:	The Jackson Laboratories, Bar Harbor, ME
PRINCIPAL INVESTIGATOR:	Wesley G. Beamer, Ph.D.
COOPERATING INSTITUTION:	Loma Linda University, Loma Linda, CA
PROPOSED PROJECT DATE:	September 27, 1999 through September 26, 2000

The appropriate program and administrative personnel of each institution involved in this grant application are aware of the PHS and National Science Foundation Consortium grant policies and are prepared to establish the necessary inter-institutional agreement consistent with carrying out this project.

Loma Linda University is in compliance with PHS and NSF policies regarding Animal Care and Use, Human Subjects, civil Rights, Handicapped Individuals, Sex Discrimination, Scientific Fraud (Misconduct) Assurance, Delinquent Federal Debt, Debarment and Suspension, Drug-Free Workplace and Lobbying.

Cooperating Institution

By: _____

Date: 9/28/99

David J. Baylink, M.D.
Principal Investigator
Typed Name and Title

By: _____

Date: 10/5/99

J. Patrick Yhip, C.P.A.
Director, Grants Management, LLU
Typed Name and Title

Applicant Institution

By: _____

Date: 10/27/99

Cookie Willems, CRA
Mgr., Grants Management
Typed Name and Title

By: _____

Date: 10/27/99

Wesley G. Beamer, Ph.D.
Principal Investigator, TJL
Typed Name and Title

COST ELEMENTS			Fourth Year (Hours)
a. DIRECT LABOR COSTS			
Wesley G Beamer	P.I.	salary	3,561
Total Salaries			3,561
b. FRINGE BENEFITS AND INDIRECT COSTS			
Employee Benefits			1,033
Indirect Costs			18,378
TOTAL			19,411
c. MAJOR EQUIPMENT			0
d. MATERIALS, SUPPLIES, AND CONSUMABLES			
e. SUBCONTRACTS			
	Direct		67,660
	Indirect		18,268
			85,928
f. TRAVEL COSTS			
g. PUBLICATION AND REPORT COSTS			
h. CONSULTANT COSTS			0
i. OTHER DIRECT COSTS			
			0
TOTAL EXPENSES			108,900

ANNUAL REPORT - year 3

INTRODUCTION

Osteoporosis and the importance of peak bone density

Osteoporosis is a disease of insufficient bone density. In the human skeleton, bone density increases with age, reaching peak levels at 18-22 years of age. As bone density decreases with age, the risk of osteoporotic fractures increases, especially when the density falls below the fracture threshold. This relationship suggests that the risk of osteoporotic fracture can be defined in terms of two characteristics - the peak bone mineral density (BMD) achieved and the net bone loss rate. The subject of this application is peak BMD, since theoretically, a person with a higher peak BMD would be less likely to fall below the fracture threshold than a person with a lower peak BMD, if both lost bone at a similar rate. During the past several decades, considerable progress has been made in understanding the pathogenesis of osteoporosis with respect to cellular mechanisms that account for age-related bone loss, but we still know very little about the mechanisms that determine peak BMD. This gap in our understanding has become critical because of recent evidence that as much as 70% of the variation in peak BMD can be accounted for by genetic factors. To identify such heritable factors in humans, investigations have primarily focused on unrelated populations to find association of BMD and restriction fragment length polymorphisms (RFLPs) in genes thought to be involved with BMD. These population studies are subject to sampling bias, lack of knowledge about how many alleles there are, lack of knowledge about parental sources of genetic alleles, and have low statistical resolving power.

Our proposal represents the first attempt to use QTL mapping for the analysis of bone density in B6C3F2 mice. The major problem was not the genetic infrastructure, but the need for reliable methodology for measuring bone density that could be applied to large numbers of mice. This methodological problem has been solved with the peripheral quantitative computerized tomography (XCT 960M) described in Beamer et al. (Bone 18:397, 1996). Once major genes have been identified by the first QTL mapping cross (i.e., B6C3F2 mice), further statistical and genetic techniques are able to isolate and study the individual genes.

BODY OF STUDIES CONDUCTED DURING YEAR 3

Specific Aim 1 (Conducted at The Jackson Laboratory, Bar Harbor, ME). **To map genes that regulate the difference in peak bone density between C3H/HeJ and C57BL/6J mice.** The location of genes responsible for the differences in peak bone density between C3H/HeJ and C57BL/6J strains will be accomplished through combination of genetic crosses and molecular analytical methods.

The genetic crosses utilized in segregating bone density regulatory genes for QTL mapping are: **a**) F2 progeny from intercrosses of (C57BL/6J x C3H/HeJ)F1 parents, **b**) BXH Recombinant Inbred (RI) strain progeny derived from C57BL/6J and C3H/HeJ progenitors; and, **c**) Congenic strains derived by repeated cycles of backcrossing C3H/HeJ 'donor' genes into the C57BL/6J 'background'. To gain additional BMD QTLs via matings with another high BMD inbred strain, we have: **d**) mapped genetic loci in a second F2 intercross progeny set from (C57BL/6J x CAST/EiJ)F1 parents, and **e**) analyzed Congenic strains derived by repeated cycles of backcrossing CAST/EiJ 'donor' genes into the C57BL/6J 'background'. The BMD phenotypes are assessed by peripheral quantitative computerized tomography (pQCT).

Genotyping is accomplished by scoring DNA for molecular markers (simple sequence length and cDNA polymorphisms) that identify genetic alleles originating from either C57BL/6J (B6), C3H/HeJ (C3H), or CAST/EiJ (CAST) strains. Co-segregation of molecular markers with BMD establishes the genetic linkage to specific chromosomal regions, the number of genes involved in bone density

differences, the location of bone regulatory genes with strong and modifier effects, the mode of inheritance for each gene, the order of the bone density genes with respect to flanking loci, and estimates of variance in BMD accounted for by each locus.

Progress

a) Provision of mice for research projects. Inbred and hybrid mice used in **Aims 1** were raised in Dr. Beamer's animal colony space at The Jackson Laboratory, Bar Harbor, ME. Colonies of B6, C3H, B6C3F1, B6C3F2, CAST mice have been the subjects for genetic and endocrinologic analyses carried out in Bar Harbor, ME. Approximately half of the B6 and C3H mice utilized for biochemical and cell biology analyses carried out in Loma Linda, CA, were raised in Dr. Beamer's research colonies, while the remaining half of the mice were purchased from the Animal Resources Colonies of the Jackson Laboratory in Bar Harbor. This collaboration will continue for remaining period of support, and it maximizes the efficient use of mice and facilitates interpretation of research results.

b) Phenotype analyses - femurs and vertebrae. Our goal is to locate the genes responsible for the 50% difference in peak BMD between B6 versus C3H and the 35 % difference between B6 versus CAST mice. We have conducted studies to ascertain the best phenotype upon which to base genetic analyses of bone density in mice. Femurs from young adult B6, C3H, and CAST females at 4 months of age were measured by pQCT (XCT-960M, Norland Med Sys., Ft. Atkinson, WI), by histomorphometry of undecalcified mid-diaphyseal shaft sections, and by μCT-20 (Scanco Med, Zurich). The pQCT was found to accurately measure the mineral with precision. The XMICE v5.1 software calculations tend to underestimate the endosteal surface circumference and thus over estimate the cortical cross-sectional area, most likely due to partial volume effects. Thus, the high density bone area was consistently estimated to be larger than either the histomorphometry or the μCT indicated, and the calculations for density of the cortical shell are underestimated. The periosteal measurements produced by the XCT-960M and histomorphometry are highly correlated ($r = 0.91$) and yield values within 6% of each other. Therefore, we conclude that the best phenotype for genetic analyses is total BMD obtained by dividing the femur total mineral by the total volume (mg/mm^3). Studies with vertebrae yielded a similar conclusion, i.e., total BMD represents a precise and accurate phenotype.

Developmental studies of femoral BMD in the progenitor strains - B6, C3H, and CAST - showed that adult skeletal peak BMD was established at 4 months. Therefore, F2 mice were necropsied at 4 months for serum, body weights, kidneys and skeletal specimens. Femoral and vertebral total BMD were measured by pQCT (XCT 960M), yielding the following normal distributions indicative of phenotypes with polygenic regulation.

Figure 1A. Distributions of femoral BMD in female mice from two separate intercross progenies.

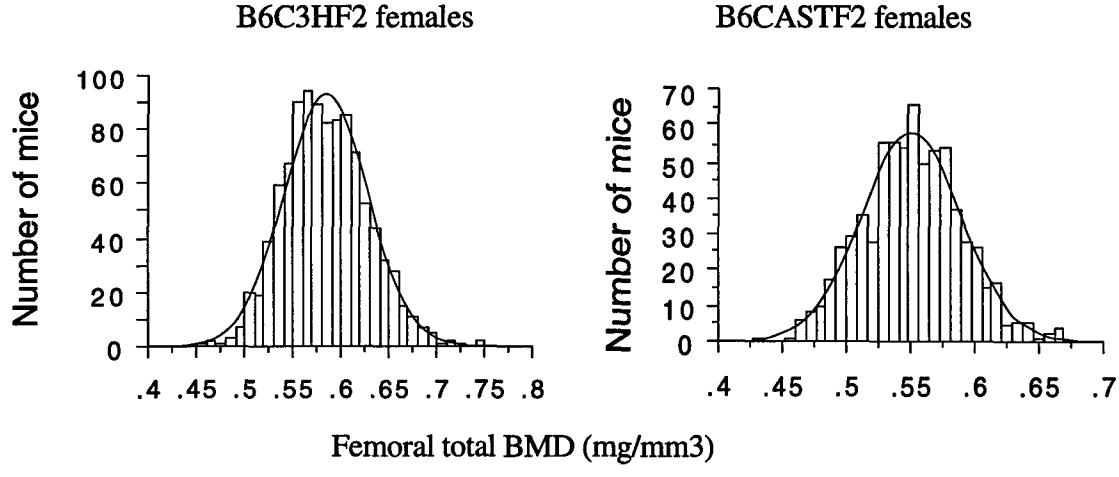
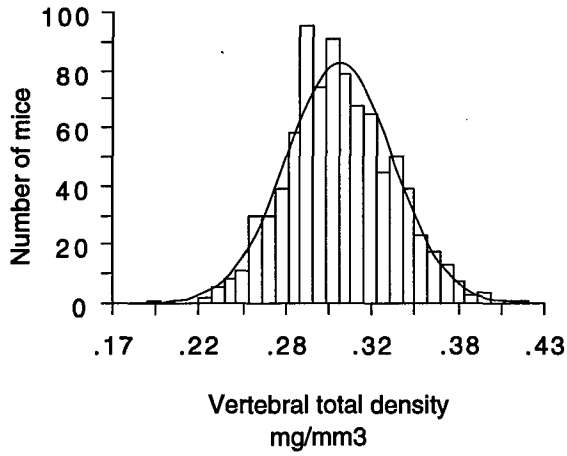


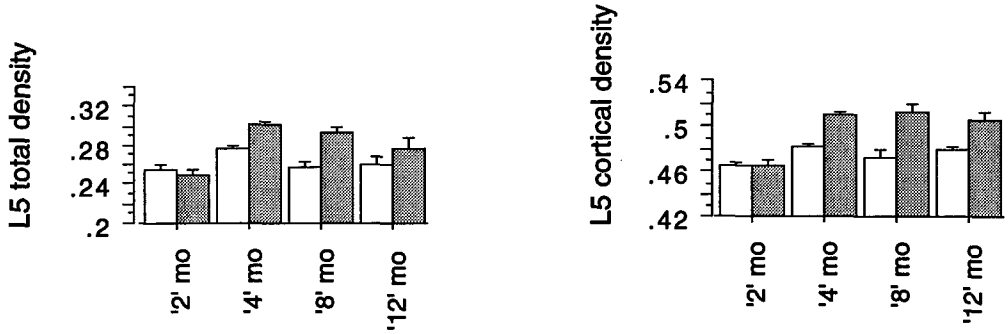
Figure 1B. Distribution of vertebral BMD in female mice from B6C3HF2 females.



Although 963 B6C3HF2 mice have been measured for vertebral BMD and 860 (89%) of these mice have been analyzed and are in the distribution presented above, the measurement of B6CASTF2 vertebrae has not yet been accomplished.

We have completed a developmental study of B6 and C3H vertebral BMD, wherein it is apparent that B6 mice compared to C3H mice have lower L5 BMD from 4 months of age onward. This pattern is consistent with that for the femur, although the difference in vertebral density is not as great. The data are presented in **Figure 2** below.

Figure 2. Total and cortical density of lumbar vertebrae for inbred progenitor strains B6 and C3H. Shaded bars = C3H/HeJ; open bars = C57BL/6J; n = 7-12 females/group; mean ± SEM.



c. Genetic analyses. Genes responsible for the difference in peak BMD are being mapped via QTL analyses of 1000 female B6C3HF2 and 714 B6CASTF2 female progenies. DNA from each F2 mouse was genotyped by Polymerase Chain Reaction (PCR) assays for polymorphic markers carried in genomic DNA of each mouse. Analyses of chromosomal regions co-segregating with high or low BMD was undertaken.

The smaller B6CASTF2 progeny set was completed during the 03 year of support and the manuscript entitled "Quantitative Trait Loci for Bone Density in C57BL/6J and CAST/EiJ Inbred Mice" will appear in Mammalian Genome in late 1999. The summarized results for the femoral total BMD analyses were: **a**) Genome wide scans for co-segregation of genetic marker data with high or low BMD revealed loci on 8 different chromosomes, four of which (Chrs 1, 5, 13, and 15) achieved conservative statistical criteria for suggestive, significant or highly significant linkage with BMD; **b**)

none of the loci exhibited significant interaction effects by ANOVA after genome wide correction for multiple tests; **c**) four BMD QTLs have been named *Bmd1* (Chr 1), *Bmd2* (Chr 5), *Bmd3* (Chr 13), and *Bmd4* (Chr 15); **d**) additive effects were observed for *Bmd1, 2 and 4,* and dominant effects for *Bmd3* ; and **e**) three of the mouse QTLs (Chrs 1, 3, 13) share linkage homology with regions on human Chr 1q21-q42, while two QTLs (Chrs 3, 15) share linkage with human Chr 8q. These homologous relationships strongly suggest that genes essential to peak BMD reside in these human chromosomal regions. Development of candidate genes awaits critical fine mapping data.

The B6C3HF2 mapping data has been completed for 200 mice in the extreme tails of the femur BMD distribution and is approximately 65% complete for all remaining B6C3HF2 mice. The genome-wide mapping analyses indicate as many as 9 loci on 8 chromosomes are correlated with femoral BMD. On the other hand, mapping data for vertebral (lumbar 5) total BMD indicated 6 loci on 6 different chromosomes are correlated with femoral BMD. Loci on Chrs 1, 4, 14, and 18 appeared shared by femurs and vertebrae for these distinct bone specimens and may represent skeletal wide genetic regulation. Unique association with BMD was found for femurs (Chrs proximal 1, 6, 11, 13, and 16) and for vertebrae (Chrs 7 and 9), representing site specific genetic regulation. **Table 1.**

Table 1. Genome wide analyses for loci associated with femoral and vertebral BMD in B6C3HF2 female mice.

	femur		vertebra L5		
Genetic locus	Likelihood Ratio Stat	Signif. level	Genetic locus	Likelihood Ratio Stat	Signif. level
D1Mit282	44.3	1.1×10^{-10}	---	no 'hit	
D1Mit14	97.3	3.2×10^{-21}	D1Mit14	52.3	4.4×10^{-12}
D4Mit124	74.7	3.3×10^{-17}	D4Mit 124	76.2	2.9×10^{-17}
D6Mit150	21.0	2.7×10^{-5}	D6	no 'hit'	
D7	no 'hit'		D7Mit332	19.7	5.3×10^{-5}
D9	no 'hit'		D9Mit196	21.0	2.7×10^{-5}
D11Mit242	29.2	4.5×10^{-7}	D11	no 'hit'	
D13Mit13	26.3	2.0×10^{-6}	D13	no 'hit'	
D14Mit160	17.3	1.0×10^{-4}	D14Mit160	14.3	8.0 x 10-4
D16Mit12	20.0	4.6×10^{-5}	D16	no 'hit'	
D18Mit36	60.3	8.1×10^{-14}	D18Mit36	31.9	1.2×10^{-7}

d) Congenic Strains. Analyses of Recombinant Congenic strain progeny was proposed as a strategy for follow-up testing of putative chromosomal regions associated with BMD. A simpler, less complex alternative is that of congenic strains, each of which carries a single chromosomal region-containing a density regulatory locus donated from another strain.

Based upon BMD loci identified in femur studies summarized in **Table 1**, we developed five B6.CAST and six B6.C3H congenic strains. These congenics were created by crossing either CAST (Chr 1, 3, 5, 13, and 14) or C3H (Chr 1, 4, 6, 11, 14, 18) chromosomal regions into the B6 strain with 6 cycles of backcrossing (N6). CAST or C3H chromosomal segments were followed by flanking polymorphic Mit markers during the backcrossing procedure. Then N6F1 mice were intercrossed to generate N6F2 mice that were genotypically homozygous for CAST versus B6 or C3H versus B6 in the donated genetic segments (heterozygous progeny were not retained for testing). These mice were raised to 4 months of age, then necropsied for tissue and bone specimens. The results for femoral BMD are presented in **Table 2A** and **2B** below.

Table 2A. Femoral data for B6.CAST congenic strains at 4 months of age (alleles: b6 = C57BL/6J, ca = Castaneus; n = 11-18 per group).

Strains	Alleles present	Body wt (g)	Length (mm)	Total dens (mg/mm3)	Cortical dens (mg/mm3)	Periosteal circum.
B6.CAST-1	ca/ca	20.7 ± 0.5	15.5 ± 0.1	0.536 ± 0.007***	0.657 ± 0.006**	4.841 ± 0.041**
	b6/b6	21.8 ± 0.8	15.4 ± 0.2	0.496 ± 0.006	0.631 ± 0.004	4.690 ± 0.039
B6.CAST-3	ca/ca	21.1 ± 0.2	15.2 ± 0.1	0.508 ± 0.005*	0.645 ± 0.004***	4.807 ± 0.030**
	b6/b6	20.8 ± 0.5	15.3 ± 0.1	0.494 ± 0.003	0.625 ± 0.003	4.646 ± 0.036
B6.CAST-5	ca/ca	21.9 ± 0.6	15.4 ± 0.2	0.455 ± 0.006**	0.610 ± 0.011*	4.893 ± 0.070
	b6/b6	21.9 ± 0.4	15.4 ± 0.1	0.478 ± 0.004	0.626 ± 0.003	4.833 ± 0.036
B6.CAST-13	ca/ca	21.4 ± 0.5	16.0 ± 0.1*	0.513 ± 0.005*	0.631 ± 0.005*	4.780 ± 0.041
	b6/b6	20.8 ± 0.5	15.4 ± 0.1	0.486 ± 0.006	0.617 ± 0.004	4.723 ± 0.038
B6.CAST-14	ca/ca	19.7 ± 0.3*	15.7 ± 0.1	0.513 ± 0.005**	0.631 ± 0.005*	4.689 ± 0.027
	b6/b6	21.1 ± 0.4	15.7 ± 0.1	0.486 ± 0.006	0.617 ± 0.004	4.679 ± 0.024

'p' values: * < 0.05, ** < 0.005, **8 < 0.0005

Table 2B. Femoral data for B6.C3H congenic strains at 4 months of age. (alleles: b6 = C57BL/6J; c3 = C3H/HeJ; n = 7-16 per group)

Strains	Alleles present	Body wt (g)	Length (mm)	Total dens (mg/mm^3)	Cortical dens (mg/mm^3)	Perios. circum (mm)
B6.C3H-1	c3/c3	23.2 ± 0.7**	15.3 ± 0.2	0.507 ± 0.007***	0.634 ± 0.006	4.736 ± 0.038
	b6/b6	20.3 ± 0.9	14.8 ± 0.2	0.474 ± 0.005	0.620 ± 0.008	4.729 ± 0.062
B6.C3H-4T	c3/c3	21.8 ± 0.5	15.8 ± 0.1*	0.527 ± 0.012**	0.660 ± 0.008**	5.096 ± 0.029**
	b6/b6	22.2 ± 0.7	15.4 ± 0.1	0.484 ± 0.004	0.624 ± 0.005	4.872 ± 0.058
B6.C3H-4A	c3/c3	21.6 ± 0.3	15.6 ± 0.1	0.513 ± 0.004*	0.653 ± 0.005	4.914 ± 0.040
	b6/b6	22.7 ± 0.6	15.6 ± 0.1	0.501 ± 0.005	0.648 ± 0.005	4.888 ± 0.034
B6.C3H-6	c3/c3	19.8 ± 0.5	14.8 ± 0.1	0.470 ± 0.005*	0.611 ± 0.004	4.581 ± 0.040***
	b6/b6	21.9 ± 0.7	15.3 ± 0.1	0.486 ± 0.006	0.622 ± 0.005	4.771 ± 0.032
B6.C3H-13	c3/c3	21.9 ± 0.9	15.2 ± 0.1	0.505 ± 0.006*	0.640 ± 0.009*	4.586 ± 0.048*
	b6/b6	22.0 ± 0.6	15.4 ± 0.1	0.485 ± 0.004	0.620 ± 0.002	4.740 ± 0.039
B6.C3H-18	c3/c3	21.7 ± 0.5	15.5 ± 0.2	0.501 ± 0.009	0.639 ± 0.008	4.696 ± 0.023
	b6/b6	20.6 ± 0.7	15.2 ± 0.2	0.490 ± 0.005	0.625 ± 0.008	4.669 ± 0.057

'p' values: * < 0.05, ** < 0.005, ** < 0.005

Results: *B6.CAST congenic strains.* Each of these congenic strains shows significant changes in femoral total BMD and in cortical BMD. CAST alleles resulted in changes in Chr 1 (8.1%), 3 (2.8%), 5 (-4.8%), 13 (3.3%), and 14 (5.5%). Only B6.CAST-5 carrying CAST alleles showed loss of BMD.

Significant changes were also found for each congenic strain in cortical BMD. It is interesting that summation of the absolute percent change in total BMD yields about 2/3 of the net difference in total BMD for the B6 versus the CAST strains (~35%).

Results: *B6.C3H congenic strains.* Four of the 5 B6.C3H congenics showed significant changes in percent femoral total BMD: Chrs 1 (7.0%), 4T (8.9%), 4A (2.4%), 6 (-3.3%), 13 (4.1%), and 18 (2.2%). Changes in cortical density were always in the same direction as total BMD, but only changes in B6.C3H-4T and B6.C3H-13 were significant. Again, C3H alleles increased BMD except in B6.C3H-6 where C3H alleles decreased total BMD. The congenic strains for Chr 11 and 16 have not yet achieved N6 cycles of backcrossing; data will not be available until Spring 2000. These congenic strains are being expanded to provide mice to Dr. Baylink and associates in Loma Linda for initial studies of bone formation and resorption.

In summary, the more complex RC strain preparation is not necessary for analyses of single gene effects on bone density. Instead, the simple congenic strain system will allow a) fine genetic mapping leading to cloning of these genes, b) provide virually unlimited numbers of mice to conduct the biological studies that will assist in identifying what these genes do, and c) serve as possible tools for drug discovery aimed at exogenous manipulation of bone density.

New Instrumentation - PIXImus DEXA. We have been testing a dual energy X-ray absorption instrument capable of acquiring body composition {mineral, fat, and lean (protein + water)} and skeletal areal BMD in mice. The PIXImus is a small animal DEXA instrument (LUNAR, Madison, WI) that has been reconfigured from the existing instrument designed to measure human calcaneus areal density, named the Peripheral Instantaneous X-ray Imager (PIXI). The PIXImus has reduced X-ray energies to achieve contrast in small bones and tissues with lower densities. Standard operation utilizes a resolution of 0.18 x 0.18 mm pixels for the whole body mode. Data can be collected from anesthetized mice in 5 minutes, allowing for repeated studies on the same mice over time. The precision for areal BMD is excellent, less than 1% for whole body and approximately 1.5% for specialized regions (e.g., spine, femur).

The following data compare: **a**) body composition data obtained by PIXImus with archival biochemical extraction data (**Table 3**); **b**) vertebral areal BMD obtained by PIXImus with volumetric BMD obtained by pQCT (**Figure 3**); and c) QTL mapping of the PIXImus vertebral areal BMD phenotype with pQCT vertebral volumetric BMD phenotype (**Table 4**).

Table 3. Comparison of Body Composition Data; Biochemical Extraction versus PIXImus DEXA determinations. Mice were C57BL/6J-*lit/lit* and *lit/+* , Males and Females 8 Weeks of Age. *lit* mutation is defective Ghrhr and causes isolated GH/IGF-I deficiency in homozygous mutants.

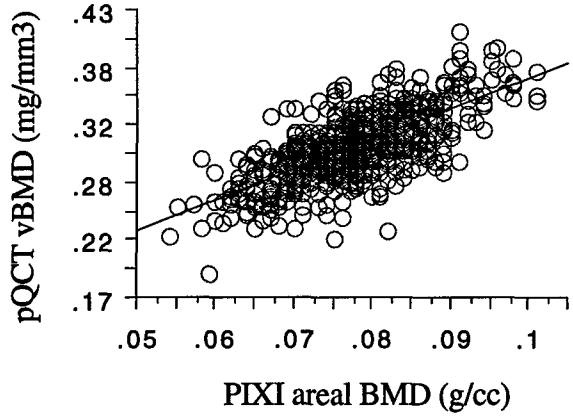
Mouse genotype	lean (g) Extraction	lean (g) PIXImus	fat (g) Extraction	fat (g) PIXImus
lit/+ males (n=6-8)	14.8 ± 0.6	14.3 ± 0.3	3.0 ± 0.1	4.1 ± 0.3
lit/+ females (n=6-10)	11.6 ± 0.4	11.1 ± 0.3	2.5 ± 0.1	3.0 ± 0.1
lit/lit males (n=6-11)	7.0 ± 0.1	7.2 ± 0.4	3.3 ± 0.2	3.4 ± 0.4
lit/lit females (n=7-11)	6.4 ± 0.2	6.5 ± 0.1	2.5 ± 0.2	2.4 ± 0.1

Table 3 (continued). Comparison of Body Composition Data; Biochemical Extraction versus PIXImus DEXA determinations. Mice were C57BL/6J-*lit/lit* and *lit/+* , Males and Females 8 Weeks of Age. *lit* mutation is defective Ghrhr and causes isolated GH/IGF-I deficiency in homozygous mutants.

Mouse genotype	% lean		% fat	
	Extraction	PIXImus	Extraction	PIXImus
lit/+ males (n=6-8)	81.3	77.7	16.5	22.3
lit/+ females (n=6-10)	80.6	78.7	17.2	21.2
lit/lit males (n=6-11)	66.9	68.4	31.5	31.8
lit/lit females (n=7-11)	71.2	73.5	27.5	29.0

The above data in **Table 3** validate the accuracy of the PIXImus body composition data by showing that both the absolute amount of mass detected and the percent of total mass are statistically not different from data determined by biochemical extraction published previously (Donahue & Beamer, 1993, J. Endocrinol. 136:91). This now permits incorporation of changes in lean tissue with changes in BMD as a function of treatment conditions and with new studies on genetics of bone strength .

Figure 3. Comparison of BMD obtained by pQCT and by PIXImus for 614 B6C3HF2 female mice.



In **Figure 3** above, we found that the areal and volumetric BMD values were significantly correlated ($r = 0.704$; $P < 0.001$) for the same group of 614 B6C3HF2 female mice. We found that bone mineral content correlated even better than BMD ($r = 0.902$; $p < 0.0001$), indicating that the major difference in absolute values for BMD were related to the different denominators. Again, these data confirm that the PIXImus is accurately measuring skeletal mineral in specimens as small as mice.

Vertebral mapping data obtained with the PIXImus phenotype data are shown in **Table 4** below.

Table 4. Comparison of chromosomal regions from analyses of vertebral BMD phenotypes obtained from pQCT (L5) and from PIXImus (L2-4) X-ray based densitometry instruments.

PIXImus			pQCT		
Genetic locus	Likelihood Ratio Stat	Signif. level	Genetic locus	Likelihood Ratio Stat	Signif. level
D1Mit14	33.6	5.0×10^{-8}	D1Mit14	52.3	4.4×10^{-12}
D4Mit124	52.3	4.4×10^{-12}	D4Mit 124	76.2	2.9×10^{-17}
D7	no 'hit'		D7Mit332	19.7	5.3×10^{-5}
D9	no 'hit'		D9Mit196	21.0	2.7×10^{-5}
D14Mit160	26.7	1.6×10^{-6}	D14Mit160	14.3	8.0 x 10-4
D18Mit36	20.3	3.9×10^{-5}	D18Mit36	31.9	1.2×10^{-7}

Most importantly, when we examined the genome-wide genetic analyses for association of chromosomal regions with vertebral areal BMD, 4 of the 6 autosomes found in the pQCT data were also detected in the PIXImus data. Statistically, the pattern the 'p' values for each chromosomal regions was similar, although absolute 'p' levels were lower for the areal BMD data. One clear difference seen in the pQCT vertebral data was the two additional loci, one each on Chr 7 and 9, that were not detected in the areal BMD data. We suspect that this may be a function of differences in instrument resolution. At this time, however, we can not exclude the interpretation that differences in bone size interpreted as volume (pQCT) or area (PIXImus) may affect detection of differences in BMD phenotypes associated with Chrs 7 and 9.

KEY RESEARCH ACCOMPLISHMENTS

These accomplishments at The Jackson laboratory research site are:

a) 11 genetic loci regulating peak femoral BMD have been mapped in mice;
b) 6 genetic loci regulating peak vertebral BMD have been mapped in mice;
c) 8 of 9 BMD loci have been established in congenic strains and confirmed for BMD regulation;
d) new instrumentation has been established and validated for physiological and genetic studies of BMD.

REPORTABLE OUTCOMES

-manuscripts, abstracts and presentations

{all publications that follow are sufficiently new that reprints have not yet been received. Copies will be provided as soon as these arrive in Bar Harbor.}

refereed journal articles

1. Linkhart TA, Linkhart SG, Kodama Y, Farley JR, Dimai HP, Beamer WG, Donahue LR, Rosen CJ, and Baylink DJ. 1999. Osteoclast formation in bone marrow cultures from two inbred strains of mice with different bone densities. J. Bone Mineral Res. 14: 39-46.
2. Beamer WG, Shultz KL, Churchill GA, Frankel WN, Baylink DJ, Rosen CJ, Donahue LR. 1999. Identification of quantitative trait loci for bone density in C57BL/6J and CAST/EiJ inbred mice. Mamm. Genome. In press, Fall 1999.

3. Sheng, M H-C, Baylink, D J, Beamer WG, Donahue LR, Rosen CJ, Lau K-HW, Wergedal JE. 1999. Histomorphometric studies show that bone formation and bone mineral apposition rate are greater in C3H/HeJ (high density) than in C57BL/6J (low density) mice during growth. Bone. 25: 421-430.
4. Kodama Y, Umemura Y, Nagasawa S, Beamer WG, Donahue LR, Rosen CJ, Baylink, DJ, and Farley JR. 199_. Exercise and mechanical loading increase periosteal bone formation in C57BL/6J mice, but not in C3H/HeJ mice. Submitted to Bone.
5. Crawford G, Beamer WG, Rosen CJ, Baylink DJ, Richman CC, and Barker JE. 2000. Characterization and mapping of the mouse mutation Spontaneous Fracture (*sfx*): A model for defective bone formation. Bone. Accepted pending minor revisions.

abstracts

6. Donahue LR, Rosen CJ, Beamer WG. 1999. Comparison of bone mineral content and bone mineral desnity in C57BL/6J and C3H/HeJ female mice by pQCT (Stratec XCT 960M) and DEXA (PIXImus). Int'l. Mouse Genetics Mtng., Oct 1999.
7. Beamer WG, Rosen CJ, Donahue LR, Frankel WN, Churchill GA, Shultz KL. 1999. Mice congenic for bone density QTLs exhibit corresponding bone density changes. 21st Ann. Mtng. Amer. Soc. Bone & Mineral Res., St. Louis, MO.
8. Richman C, Kutilek S, Myakoshi K, Beamer WG, Donahue LR, Rosen CJ, Wergedal J, Baylink D. 1999. Evidence that prepubertal and pubertal skeletal changes contribute to the extreme differences in peak bone density (BD) between C3H/HeJ (C3) and C57BL/6J (B6) strains of mice. 21st Ann. Mtng. Amer. Soc. Bone & Mineral Res., St. Louis, MO.
9. Mohan S, Wergedal J, Kutilek S, Kodama Y, Beamer WG, Baylink D. 1999. Site specific effects of PTH (1-34) and PTH (1-31) analogs on bone formation in mice: Implications on bone strength. 21st Ann. Mtng Amer. Soc. Bone & Mineral Res., St. Louis, MO.
10. Rosen CJ, Friez J, Donahue LR, Shultz KL, Beamer WG. 1999. Determination of a major quantitative trait locus (QTL) of IGF-I in mice: Association with peak bone mass and implications for identification of potential IGF-I regulatory genes. 21st Ann. Mtng Amer. Soc. Bone & Mineral Res., St. Louis, MO.
11. Exercise and mechanical loading increase perisoteal bone formation in C57BL/6J (B6) mice, but not in C3H/HeJ (C3H) mice. 1999. Kodama Y, Umemura Y, Nagasawa S, Farley J, Beamer WG, Donahue LR, Rosen CJ, Baylink DJ. 21st Ann. Mtng Amer. Soc. Bone & Mineral Res., St. Louis, MO.

- **patents & licenses applied for and/or issued**: None

- **degrees obtained that are supported by this award**: None

- **development of cell lines, tissue or serum repositories**: None
- **informatics such as data bases and animal models**: 11 congenic strains carrying BMD regulatory loci established

- **funding applied for based upon work supported by this award**: Application to Genetics Institute, Andover, MA, for support of "Identification and functional characterization of genes associated with regulation of peak bone density and investigation of sfx mutant mice."

- **employment or research opportunities applied for and or received on expreiences/training support by this award**: None

CONCLUSIONS.

The QTL mapping strategy works exceptionally well for discovery of BMD regulatory loci and

their mode of action. At least 9 loci have been found that account for the difference in bone density between B6 and C3H mice, and at least 5 loci account for differences between B6 and CAST/EiJ. Femoral and vertebral bones have both similar and unique genes. Eight of 9 congenic strains developed to test effects of individual loci on bone density and to assist with fine mapping have proven that the BMD regulatory loci have independent effects; two ongenics are still in progress of development. Biological studies of these congenic strains are beginning that will indicate whether whether bone formation or bone resorption are the processes most affected by each BMD allele. Determining precisely where these genes are and what bone physiology mechanisms they regulate remains our overall goal. The outcome of such studies will also provide new models for pharmacologic intervention in repair of bone quality and density.

These genetic loci regulating BMD in mice are those that regulate normal variability in BMD, rather than mutations with bone pathology consequences. Such mapping and biological studies can only be done in mice, where precise control of matings and environment can be exercised. The power of detection and creation of models to define the biological systems affected, as well as to define gene-gene and gene-environment interactions can not be duplicated by association studies in humans. Finally, these genetic mapping data in mice can be used to predict the location of similar BMD regulatory genes in the human genome by comparative mapping data bases.

REFERENCES

None required.

Progress Report of Work Performed at Loma Linda University

This section summarizes the progress of the work performed at Loma Linda University during the third year of the funding. To facilitate the understanding of our progress, this section will be subdivided into three sections: In the first section, we will discuss our progress toward the original specific aim (aim 2) of the grant proposal. The second section focuses on progress toward goals that are not included in the original proposal but are relevant to the overall objective of the project. The last section will address what we intend to accomplish during year 4.

I. Progress toward specific aim 2. Our overall objective in this specific aim is to identify bone modeling (i.e., bone formation and bone resorption) mechanisms that characterize the skeletal phenotypes of C57BL/6J (B6) and C3H/HeJ (C3H) mice. Accordingly, there were three major focuses in our work during the year 3 of this project: 1) to complete measurements on trabecular bone histomorphometric parameters of bone formation and resorption in tibia and femur by further analyzing osteoblastic (bone forming) and osteoclastic (bone resorbing surfaces), 2) to extend these studies to the vertebra of C57BL/6J, C3H/HeJ mice in order to determine if differences in the vertebra between mouse strains are similar to the differences in the long bones, and 3) to continue to develop and apply serum markers of bone formation and resorption.

A. ***Bone histomorphometric measurements.***

1. Brief summary of previous work. We previously reported that differences in BFR and/or MAR between C3H and B6 mice are detected at both periosteal as well as endosteal bone sites. The differences in bone area and BFR are also evident at tibia as well femur. Consequently, these observations are entirely consistent with the contention that the strain-associated difference in bone formation and bone mass is not site-specific. These findings are consistent with our previous observations that the strain-related difference in bone density was evident in the vertebra, proximal phalanges, tibia, as well as femur. The differences in the bone mineral apposition rate (MAR) are particularly interesting because they suggest that the difference between the C3H and B6 is in the activity of the osteoblasts. To further investigate the differences between the C3H and B6 mice we have examined trabecular bone formation in the distal femur metaphysis. In the initial studies given in the last report and summarized below in Tables 1 and 2 we provided evidence that the differences in bone formation and bone apposition between C3H and B6 were also present in trabecular bone. The methodology for these observations is given in 2. Below.

2. Methodology: Before we describe our results thus far, we will briefly summarize the methodology that we had developed for measurements of metaphyseal parameters. Briefly, the distal end of femur (or proximal end of the tibia) were dehydrated in 95% and 100% ethanol, respectively, two days each. After the dehydration, the bone specimens were infiltrated with a mixture containing 85 ml methyl methacrylate, 10 ml glycol methacrylate, 5 ml dibutyryl phthalate, 5 ml polyethylene glycol (PEG 600), and 0.9% dried benzoyl peroxide (as a catalyst) for 4-6 days. Polymerization was initiated by mixing 1 ml of JB-4 solution and 100 ml of the above infiltration solution and was carried out in plastic molding cups (6 x 12 x 5 mm) with the

infiltrated specimens placed at the bottom and aluminum blocks placed on top. Each bone specimen was orientated at a position with the anterior part of the femur facing down. To get complete penetration and polymerization, all molding cups were placed in an anaerobic jar and the air removed with vacuum followed by flushing with nitrogen for 5 to 10 min.

Five μm thin bone sections were cut with a Reichert-Jung 1140 Autocut microtome (Buffalo, New York). During sectioning, the knife and tissue blocks were kept wet with 40% ethanol. The resulting sections were either: a) stained with Goldner's for analysis of trabecular bone volume, b) stained histochemically for tartrate-resistant acid phosphatase (TRAP) activity to identify osteoclasts, or c) analyzed unstained for bone formation parameters. All bone histomorphometric parameters were measured with the OsteoMeasure™ system on images captured with a color video camera system equipped with a digitizing tablet (OsteoMetrics, Inc., Atlanta, GA). The sampling site was 0.3 mm down from the growth plate; and the size of the measurement area was between 0.8 to 0.9 mm^2. Total tissue area, trabecular bone area, trabecular bone perimeter, tetracycline labeled length, and the width of double tetracycline labels were measured. Trabecular bone volume, thickness, number, mineral apposition rate (MAR), tetracycline labeled surface and total and relative bone formation rate (BFR) were then calculated.

Table 1. Structural parameters of trabecular bone in C3H and B6 mice.

	Tissue area (mm^2)	Bone area (mm^2)	Tb volume (%)	Tb thickness (μm)	Tb number (#/mm)	Tb separation (μm)
6 weeks						
C3H	0.529±0.027	0.155±0.012	30.07±3.32	40.59±3.40	7.409±0.462	98±9
B6	0.582±0.039	0.112±0.012	19.53±1.71	30.09±1.61	6.516±0.496	129±12
12 weeks						
C3H	0.817±0.027	0.144±0.012	17.48±0.96	39.27±1.51	4.448±0.158	187±8
B6	0.812±0.019	0.087±0.010	10.64±1.13	26.34±1.52	4.026±0.373	232±21
P						
Strain	n.s.	<0.001	0.001	<0.001	n.s.	0.008
Age	<0.001	n.s.	<0.001	n.s.	<0.001	<0.001
StrainxAge	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

3. Comparison of trabecular bone formation parameters between C3H and B6 mice.

a. *Trabecular bone volume.* Table 1 shows that C3H mice also had a significantly greater trabecular bone volume than B6 mice did 32% greater at 6 weeks old and 64% at 12 weeks old. While trabecular bone volume appeared to decline with age in both mouse strains, the effects of strain (P=0.040) and age (P=0.002) were highly significant. However, the larger trabecular bone volume seen in C3H mice was not a result of a larger number of trabeculae (since there was no significant difference in the number of trabeculae between the two mouse strains at both 6- and 12-week old. Rather, it was due to a larger trabecular thickness since C3H mice had a 16% and 49% larger trabecular thickness at 6 weeks and 12 weeks old, respectively.

b. *Trabecular bone formation parameters.* Table 2 compares bone formation parameters of trabeculae between C3H and B6 mice of 6 and 12 weeks old, respectively. BFR rate in trabeculae in C3H, like in cortical bone, was significantly (P=0.006, ANOVA) higher than that in B6 mice of 6 week (23%) or 12 week (68%) of age. Correspondingly, MAR was also 29% and 23% higher (P=0.001, ANOVA) in C3H mice of 6 and 12 weeks old, respectively, than in B6 mice of

Table 2. Bone formation parameters in trabecular bone of C3H and B6 mice.

	Total longitudinal section referent		Bone surface reference		
	BFR (mm^2x10^{-3}/day)	TLS (mm)	BFR/BS (mm^2x10^{-3}/mm/day)	MAR (μm/day)	TLS/BS (mm/mm)
6 weeks					
C3H	5.291±0.946	3.646±0.574	0.757±0.144	2.357±0.101	0.514±0.073
B6	3.390±0.549	3.060±0.378	0.446±0.067	1.894±0.103	0.402±0.044
12 weeks					
C3H	6.822±0.805	3.807±0.361	1.111±0.085	2.611±0.133	0.629±0.053
B6	4.049±0.613	2.846±0.281	0.753±0.128	2.128±0.122	0.549±0.086
P					
Strain	0.005	n.s.	0.005	<0.001	n.s.
Age	n.s.	n.s.	0.006	0.044	n.s.
StrainxAge	n.s.	n.s.	n.s.	n.s.	n.s.

n.s., not significant, P>0.05

corresponding age. Conversely, TLS in trabeculae was not significantly different in either strains of either age. These findings suggest that, like in cortical bone, C3H mice has a higher BFR in trabecular bone than B6 mice, and that the increased trabecular bone formation appeared to be caused by an increased osteoblast activity rather than an increased osteoblast proliferation. Thus increased osteoblastic activity was characteristic of the trabecular bone surfaces of the metaphysis as we have previously shown for the endosteal and periosteal surfaces.

c. *Osteoblast and osteoclast parameters in trabecular bone of C3H and B6 mice at 6 weeks of age*. Our studies of trabecular bone have shown that trabecular bone volume is higher in C3H mice than in B6 mice consistent with the higher bone density of the C3H mice. This higher bone volume is associated with increased bone formation that is primarily due to a higher rate of bone matrix apposition (MAR) in the C3H mice. These findings in trabecular bone are similar to those we have previously shown to be present on periosteal and endosteal surfaces. Thus, it was important to identify the mechanism of the increased apposition. Because the methyl methacrylate embedding used in the trabecular bone analysis has good cell preservation, it allows more detailed analysis of osteoblast parameters than is available in the cortical bone analysis. Increases in the bone matrix apposition rate could be due to two different processes, 1) due to increased bone formation activity per osteoblasts, or 2) to increased number of active osteoblasts per unit surface. Therefore, osteoblast numbers were determined in metaphyseal sections from 6-week-old mice. All cells that showed osteoblast characteristics and were present on a forming surface identified by the presence of osteoid were counted and the forming surface length measured. In addition, demineralized paraffin embedded sections were prepared from the opposite femur for analysis of osteoclast parameters using acid phosphatase activity to identify the osteoclasts.

Table 3. Osteoblast and osteoclast parameters in trabecular bone of C3H and B6 mice at 6 weeks of age.

	C3H	B6	P
Osteoblast Parameters			
Ob.no	262±26 (8)	205±36 (7)	NS
Ob.no/Od.pm	106.2±4.0 (8)	118.0±4.4 (7)	0.070
Ob.no/Tb.pm	40.35±3.12 (8)	32.76±5.80 (7)	NS
Od.pm/Tb.pm	0.385±0.037 (8)	0.283±0.058 (7)	NS
DL	0.743±0.205 (7)	0.514±0.112 (7)	NS
DL+.5sL/Tb.pm	0.313±0.052 (7)	0.253±0.017 (7)	NS
OA/BA,%	7.099±1.198 (8)	5.196±1.401 (7)	NS
Od.wi	3.488±0.364 (8)	2.700±0.214 (7)	0.089
MAR	2.357±0.101 (7)	1.905±0.111 (7)	0.014
Osteoclast Parameters			
OC.no	74±6 (5)	63±4 (5)	NS
Oc.pm	2.266±0.355 (5)	1.582±0.195 (5)	NS
Oc.no/Tb.pm	9.833±0.611 (5)	8.627±0.609 (5)	NS
Oc.pm/Tb.pm	0.301±0.040 (5)	0.213±0.023 (5)	NS

NS, P>=1.000

These studies showed that the bone forming surface measured by either the presence of osteoid or the presence of a tetracycline label was higher in the C3H mouse strain than in the B6 strain (Table 3) although these differences did not reach statistical significance. The number of osteoblasts per section was also higher in the C3H mice. However the number of osteoblasts per mm of forming surface was not higher in the C3H mouse. If anything the value was lower than in the B6 mice although this did not reach statistical significance. This result indicates that the high bone matrix apposition rate in the C3H mice is not due to increased numbers of osteoblast per unit forming surface. Therefore the high rate of bone matrix apposition must be due to increased bone matrix synthesis per osteoblast.

d. *Osteoclast parameters in trabecular bone.* We have previously reported evidence that a lower bone resorption rate in the C3H mice in cortical bone of the femur and tibia and in trabecular bone of the humerus is partly responsible for the higher bone volume in C3H mice compared to B6 mice. In the distal femur metaphysis examined in the current study we were unable to detect a difference between the two strains of mice (Table 3).

B. ***Histomorphometric analysis of vertebral bone.*** The initial studies on bone density by pQCT analysis showed that vertebral bone density was higher in C3H mice than B6 mice. However, some preliminary measurements by microCT suggested that trabecular bone volume was actually lower in C3H than in B6 mice. This possibility would not be consistent with higher bone density in the C3H MICE. To further investigate the trabecular bone of the vertebra, samples from C3H and B6 mice have been prepared for analysis by methacrylate embedding, sectioning and Goldner's staining. Measurement of trabecular bone volume in the vertebra showed that the % bone volume in the vertebral metaphysis was lower in C3H than B6. In fact, the B6 appeared to have a higher % bone than the C3H at all time points although this did not reach statistical significance. Additional samples are being measured. The low trabecular bone volume of the

Table 4. Structural parameters of vertebral bone in C3H and B6 mice of varying age.

Age/Strain[a]	% Bone	Trabecular thickness	Osteoid Area	Osteoid Perimeter	Osteoid Width
6 weeks					
C3H	16.0±1.9	27.2±1.7	5.43±1.76	1.21±.14	1.87±.76
B6	18.01	23.5	7.06	.40	1.80
8 Weeks					
C3H	15.1±1.0	28.7±1.2	1.38±.12	.69±.09	2.08±.16
B6	22.9±2.8	30.2±1.1	0.91±.06	.47±.05	1.90±.06
16 Weeks					
C3H	19.1±3.1	37.4±2.0	0.64±.28	.38±.13	1.50±.18
B6	25.6±5.9	43.0±12.4	1.96±1.6	.36±.07	1.83±.10

[a]Statistical analysis by ANOVA did not detect any significant differences at a P<.05.

C3H is in contrast to the higher bone volumes previously found in the long bones. Interestingly, forming surface as measured by osteoid surface length did not go along with the higher bone volume in the B6 mice. The reason for the higher bone volume in the B6 mice is not clear.

Vertebral samples consist primarily of trabecular bone in contrast to the long bones (femur, tibia, humerus) previously analyzed. The high trabecular bone content may in some way contribute to the difference between bone sites.

C. ***Analysis of congenic mice.***
Bone Samples and serum samples from the initial groups of congenic mice have been received and are being processed for analysis. Insufficient numbers of samples have been received to present any data

D. ***Development of Murine biochemical marker assays of bone turnover.*** Murine biochemical markers of bone formation and resorption are essential for rapid phenotypic characterization of bone turnover in parent strains and F1 and F2 progenies. Unfortunately, there is no readily available markers assay for mouse. Accordingly, a major specific aim of this project was to develop murine biochemical marker assays of bone turnover. In the previous progress report, we have already summarized our progress in several bone formation and resorption marker assays, i.e., osteocalcin, alkaline phosphatase, osteoclastic TRAP, urine pyridinoline and deoxypyridinoline and several growth factor assays as well as mouse creatinine assay. In this report, we will summarize our continuing progress on the development of a new serum based bone resorption assay, namely, the C-telopeptide assay.

1. Development of C-telopeptide assay for mouse serum.

We have developed a C-telopeptide ELISA, by modifying a rat C-telopeptide ELISA developed earlier (Abstract # F226, ASBMR 1999), to measure bone resorption in mice serum. The ELISA procedure utilizes an affinity purified polyclonal antibody, which was generated against a linear synthetic peptide sequence DFSFLPQPPQEKAHDGGR. The antibody epitope involves a region, which is similar in rat and mouse C-telopeptide sequence. In our ELISA, 10-20 µL of mice serum, 50 µL of biotinylated C-telopeptide antibodies, and 100 µL of C-telopeptide-HRP conjugate were incubated for 2-hours in an Avidin coated microtiter plate. After competitive binding and colorimetric development, quantitation of mice C-telopeptide was achieved by comparison with synthetic peptide calibrator. Sensitivity of the ELISA was 0.1 ng/mL. The

Table 5. Serum C-telopeptide values in sham operated, ovarectomized and ovarectomized plus estrogen replacement groups of mice.

Study groups	C-telopeptide (ug/L) (Mean ± SEM)	p-value (vs Sham)
Sham	41.4 ± 2.4	
OVX	56.6 ± 3.7	0.0019
OVX /400 ug E_2	51.9 ± 4.6	0.0625

averaged interassay (n=3) variations for two controls were CV <9% and <13%, respectively.
The *in vivo* usefulness of the C-telopeptide ELISA was evaluated by measuring C-telopeptide concentrations in two groups (n=10) of ovariectomized (OVX) mice that received weekly injections of either estradiol (40 0r 400 µg/Kg body weight) or vehicle over a period of three weeks. C-telopeptide levels in OVX groups were compared with sham operated mice treated

with vehicle. OVX mice had 37% higher C-telopeptide levels compared to sham operated mice. Estradiol repletion of OVX mice resulted in lower C-telopeptide levels compared to the vehicle treated OVX group.

In addition, changes in C-telopeptide concentration correlated (r=-0.49, p=0.0021) with changes in bone mineral density measured by peripheral quantitative computed tomography (pQCT). These observations demonstrate the responsiveness of the assay to changes in bone resorption. The in vivo utility of the above ELISA is further being evaluated by assessing the affect of Alendronate treatment of ovariectomized C57BL/6N on C-telopeptide levels, which is known to modulate the bone resorption in the mouse model.

2. Manuscript submitted:

Development and application of a synthetic peptide based osteocalcin assay for the measurement of bone formation in mouse serum
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II. Progress toward goals that are not in the original specific aims but are relevant to the overall objective of the project. During the course of the study, we became interested in the hypothesis that the differences in peak bone mass seen in the two mouse strains may also be caused by a difference in their bone responses to bone remodeling stimuli. In other words, the higher BFR seen in C3H mice could be caused by a hypersensitivity of osteoblasts in this mouse strain to bone formation stimuli, such as mechanical loading, or the lower BFR seen in B6 mice is the result of inability of osteoblasts in this mouse strain to respond to bone formation stimuli. Conversely, the lower bone resorption observed in C3H mice might be due to the inability of their osteoclasts to respond to bone resorption stimuli.

We previously reported a test of this hypothesis. Immobilization of the hind limb by sciatic neurectomy decreased bone matrix apposition in the B6 mice but not in the C3H mice. Furthermore, bone resorbing surface was increased by immobilization to a greater extent in the B6 than in the C3H. These results indicate that the B6 mice were more responsive to changes in mechanical loading than the C3H. To determine if increases in mechanical loading produces the same results as decreases in mechanical loading, we have now tested the effect of increasing mechanical loading by training mice to jump.

A. ***Jump Study***

1. Methods. To increase mechanical loading, 9-week old female B6 and C3H mice (n=10-13 mice/group) were subjected to a jumping exercise (20 jumps/day, 5 days/week, to heights of 20-30 cm), for a total of 4 weeks. Control mice did not jump. Osteocalcin, alkaline phosphatase (ALP) activity, and IGF-I were measured in serum. The left tibiae were used for histomorphometry (ground cross-sections prepared at the tibio-fibular junction), and the right tibiae and femora for determinations of bone breaking strength (3-point bending).

2. Results of the jump-training of C3H and B6 mice.

As summarized in Table 6, four weeks of jump exercise had no effect on body weight, or longitudinal growth of the tibiae or the femora in either strain of mice and these parameters were similar in C3H and B6 mice. Also summarized in Table 6 is our observation that the dry weight of the tibiae was increased by 4 weeks of jumping exercise in the B6 mice (i.e., by 2-way ANOVA and non-parametric comparison). This effect was not observed with the C3H tibiae. The observations that the dry weights of femora and tibiae from C3H mice were greater than dry weights of bones prepared from B6 mice, irrespective of treatment (2-way ANOVA) were consistent with previous findings.

Table 6. Effects of jump exercise on body weight and bone size.

Parameter	B6 Mice Control	B6 Mice Jump	C3H Mice Control	C3H Mice Jump
Body Weight (g)	20.8 ± 0.5	20.7 ± 0.2	21.6 ± 0.5	21.1 ± 0.4
Tibia Dry Weight (mg)	28.6 ± 0.5	30.4 ± 0.6[a]	31.2 ± 0.6	31.9 ± 1.1
Tibia Length (cm)	1.78 ± 0.01	1.76 ± 0.01	1.80 ± 0.02	1.79 ± 0.02
Femora Dry Weight (mg)	31.2 ± 0.8	32.0 ± 2.8[b]	33.9 ± 0.8	36.0 ± 1.4
Femora Length (cm)	1.56 ± 0.01	1.56 ± 0.01	1.51 ± 0.01	1.50 ± 0.01

Body weight was measured at the end of the experiment (i.e., after 4 weeks of jump exercise, 20 times per day, 5 days per week). The length and dry weight of the tibiae and femora were measured. All data shown as mean +/- SEM (n=10-13 per group). [a] Indicates a significant effect of jumping (i.e., compared to control mice of same strain), by non-parametric analysis, $p<0.05$. [b] Indicates a significant difference, compared to C3H mice receiving the same treatment , $p<0.05$, by 1-way ANOVA. Two-way ANOVA indicates significant effects of mouse strain (i.e., C3H vs B6) on tibial dry weight ($p = 0.011$) and femoral dry weight ($p = 0.002$), but no effect of jump exercise on either tibial or femoral dry weight. Neither ANOVA (1-way and 2-way) nor nonparametric comparisons revealed any significant inter-group differences in body weight, tibial length, or femoral length.

Table 7. Effects of jump exercise on bone strength (breaking force/body weight mN/g).

Mouse Strain	Bone	Control	Jump Exercise	Effect of Exercise
C3H	Tibia	48.78 ± 1.27	51.12 ± 1.56	p = 0.258
B6	Tibia	38.75 ± 1.14[a]	42.90 ± 1.58[a]	p = 0.053
C3H	Femur	63.46 ± 1.92	68.95 ± 2.45	p = 0.09
B6	Femur	36.58 ± 1.96[a]	36.73 ± 1.70[a]	p = 0.96

Body weight ranged from 20.74 +/- 0.22 g for the B6 jump exercise group to 21.58 +/- 0.47 g for the C3H control group, with no significant inter-group differences. N = 10, 12, 13, 13, for B6 control, B6 exercise, C3H control, and C3H exercise, respectively. Breaking force of tibiae and femora determined by 3-point bending and normalized to body weight (mN/g). Data shown as mean +/- SEM. Effect of Exercise indicates the significance of a non-parametric comparison (Kruskal-Wallis one-way ANOVA). A standard (parametric) 2-Way ANOVA indicates significant effects of both mouse strain ($p<0.001$) and jump exercise ($p=0.029$) for tibia load/body weight, and a significant effect of mouse strain ($p<0.001$) but no effect of exercise ($p=0.19$) on femur load/body weight. [a] Indicates a significant difference, compared to C3H mice receiving the same treatment, $p<0.001$.

Consistent with the effect of the jumping exercise to increase the dry weight of the tibiae in B6, but not C3H mice, the breaking strength studies revealed a jump-dependent increase in the breaking strength of the tibiae (i.e., by 2-way ANOVA). These results are summarized in Table 7. The effect of the jump exercise to increase the strength of the tibiae did not reach significance when the B6 tibiae were analyzed alone (i.e., $p = 0.053$ for jump vs. control B6 mice, by Kruskal-

Wallis test). These 3-point bending studies also demonstrated significantly greater breaking strength for both the femora and the tibiae from C3H, compared to B6 mice (i.e., an effect of the strain of mouse in our 2-way ANOVAs).

As shown in Table 8, the current data (i.e., 2-way ANOVAs) confirmed our previous reports of higher levels of ALP in extracts of C3H, compared to B6, femora and tibiae, and higher levels of IGF-I in the sera of C3H, compared to B6 mice . The only observed effect of the jumping exercise on any tested parameter was a jump-dependent increase in the serum level of IGF-I in the C3H, but not the B6 mice (i.e., a 17% increase that was significant by non-parametric comparison, $p<0.05$). We should note, however, that the serum bone formation indices (e.g., ALP and osteocalcin) are systemic and, therefore, and reflect contributions from the total skeleton, whereas the local indices (i.e., bone extract ALP activities) are specific for the tibiae and the femora. Since the effect of jump exercise is presumed to be local (i.e., muscular loading on the tibiae and femora), any change in serum ALP or osteocalcin would reflect a change in the contribution of the hindlimb that was sufficient to alter the circulating total.

Table 8. Effects of jump exercise on ALP activity, osteocalcin, and IGF-I in serum and Alp activity in extracts of bone.

Parameter	B6 Mice Control	B6 Mice Jump	C3H Mice Control	C3H Mice Jump
Serum ALP (U/mL)	0.167 ± 0.010	0.147 ± 0.010	0.155 ± 0.007	0.168 ± 0.010
Serum Osteocalcin (ng/mL)	4.79 ± 0.51	5.57 ± 0.41	3.74 ± 0.40	4.21 ± 0.36
Serum IGF-I (ng/mL)	231.6 ± 19.1	211.4 ± 14.2	391.1 ± 21.8[b]	459.3 ± 21.7[b,c]
Tibial ALP (mU/mg)	4.04 ± 0.28	3.56 ± 0.22	5.03 ± 0.30	5.07 ± 0.44[a]
Femoral ALP (mU/mg)	4.12 ± 0.51	4.04 ± 0.31	4.29 ± 0.24	4.38 ± 0.44

After 4 weeks of jump training (20 times a day, 5 days a week, n = 10-13 mice/group), sera were collected to for measurements of ALP activity, osteocalcin, and IGF-I. In addition, the right tibiae and femora (which had been used for measurements of breaking strength) were extracted for measurements of ALP activity (expressed as mU/mg dry weight of bone). Data shown as mean +/- SEM. [a] Indicates a significant difference (by 1-way ANOVA), compared to B6 mice receiving the same treatment, $p<0.01$; [b] indicates $p<0.001$. [c] Indicates a significant effect of jump exercise, $p<0.05$ (by non-parametric, Kruskal-Wallis comparison). Two-way ANOVA revealed significant mouse strain-dependent differences in serum osteocalcin ($p<0.005$), IGF-I ($p<0.001$), and Tibial ALP ($p<0.001$), but no jump exercise-dependent differences in any listed parameter.

Our histomorphometric studies showed a jump-dependent increase in periosteal perimeter ($p<0.02$, by non-parametric comparison) in B6, but not C3H mice, with no effect of exercise on medullary area, endosteal perimeter, or endosteal MAR, in either strain of mice. The results of these analyses of cross-sections prepared at the tibio-fibular junction are summarized in Table 9. These histomorphometric studies also confirmed our previous findings of greater medullary area and greater endosteal perimeter, in B6, compared to C3H mice, with no difference in periosteal perimeter. The observed effect of jumping to increase periosteal perimeter (at this tibio-fibular sampling site) in B6, but not C3H mice, was associated with a similar increase in total bone area (i.e., total area within the periosteal perimeter). The jumping protocol resulted in a 3.8% increase in total bone area in the B6 mice, which was significant by non-parametric comparison. These two observations – jump-dependent increases in periosteal perimeter and total bone area in B6 but not in C3H mice – are geometrically consistent, as the perimeter defines the total area.

Table 9. Effects of jump exercise on selected histomorphometric indices.

Parameter	B6 Mice Control	B6 Mice Jump	C3H Mice Control	C3H Mice Jump
Periosteal Perimeter (mm)	3.15 ± 0.01	3.22 ± 0.02[a]	3.19 ± 0.02	3.20 ± 0.03
Endosteal Perimeter (mm)	1.91 ± 0.02	1.87 ± 0.02	1.44 ± 0.05[c]	1.46 ± 0.03[c]
Medullary Area (mm^2)	0.281 ± 0.007	0.272 ± 0.006	0.166 ± 0.008[c]	0.163 ± 0.007[c]
Cortical Area (mm^2)	0.474 ± 0.008	0.513 ± 0.017[a]	0.610 ± 0.013[c]	0.613 ± 0.015[c]
Endosteal MAR (μm/day)	1.84 ± 0.15	1.87 ± 0.13	1.18 ± 0.20[b]	1.46 ± 0.03[b]

After 4 weeks of jump training (20 times a day, 5 days a week), the mice were sacrificed to dissect the tibiae. Undecalcified ground cross sections were made at the Tibial-fibilar joint of the tibiae, followed by histomorphometric analyses. [a] Indicates a significant effect of jump exercise (i.e, by non-parametric comparison to control mice of the same strain), $p = 0.015$. [b] Indicates a significant difference, compared to B6 mice receiving the same treatment by 1-way (parametric) ANOVA, $p<0.05$; [c] indicates $p<0.001$. Two-way ANOVA indicates no effect of mouse strain or jump exercise on periosteal perimeter, and significant effects of mouse strain ($p<0.001$), but not jump exercise on medullary area, endosteal perimeter, and endosteal MAR.perimeter and total bone area in B6, but not C3H mice – are geometrically consistent, as the perimeter defines the total area.

Table 10. Effects of jump exercise on bone forming and resorbing surfaces.

Parameter	B6 Mice Control	B6 Mice Ump	C3H Mice Control	C3H Mice Jump
Periosteal Forming Surface	58.0 ± 2.1	65.2 ± 4.0	55.9 ± 1.4	54.7 ± 1.65[a]
Periosteal Resorbing Surface	28.9 ± 1.9	26.6 ± 2.1	33.4 ± 2.0	31.3 ± 2.8
Endosteal Forming Surface	67.8 ± 6.5	72.4 ± 7.1	65.4 ± 4.3	61.2 ± 2.1
Endosteal Resorbing Surface	25.3 ± 2.8	19.6 ± 3.3	30.0 ± 1.6	27.0 ± 1.4

After 4 weeks of jump exercise (20 times a day, 5 days a week, n = 10-13 mice/group), undecalcified ground cross sections, prepared at the tibio-fibilar junction, were used for histomorphometric measurements of forming and resorbing surfaces. Both resorbing (scalloped) and forming (single labeled, non-scalloped) surfaces are shown as percent of total surface length (mean +/- SEM). Absolute surface lengths may be determined by comparison with endosteal and periosteal perimeter lengths as specified in Table 4. [a] Indicates a difference (by 1-way ANOVA), compared to B6 mice receiving the same treatment, $p < 0.05$. Two-way ANOVA revealed: no significant effect of either mouse strain or jump exercise on endosteal forming surface; and significant effects of mouse strain, but not jump exercise, on periosteal forming surface ($p<0.02$), periosteal resorbing surface ($p<0.05$), and endosteal resorbing surface ($p<0.01$). Non-parametric comparisons (Kruskal-Wallis 1-way ANOVA) revealed no effect of jump exercise on any listed parameter, in either strain of mice.

Further histomorphometric analyses allowed for an assessment of the roles of bone formation and resorption as presumptive determinants of the observed, jump-dependent changes in tibial breaking strength and periosteal perimeter in B6, but not C3H mice. As shown in Table 10, we found no effects of either the jump exercise or the strain of mice (i.e., B6 vs. C3H) on the relative length of endosteal bone-forming surface. We did, however, find that B6 mice had smaller percentages of their endosteal and periosteal surfaces involved in resorption, and a larger percentage of their periosteal surface involved in bone formation, than the C3H mice. Two bone formation parameters were increased by the jump exercise (at our tibio-fibular junction sampling site) in B6, but not C3H mice. We found that the periosteal MAR was increased by 35% ($p<0.02$ by non-parametric comparison) in the jump-exercised B6 mice, but was not affected by jumping,

in the C3H mice. The rate of bone formation at the periosteum was, similarly, increased by jumping in B6, but not C3H mice.

B. ***Effect of calcium deficiency on B6 and C3H mice***

Because the two inbred strains of mice respond differently to changes in mechanical stress, we further examined whether a different stress namely, calcium deficiency affected these two mouse strains differently. We reported in the previous report the partial results of an initial study to test whether C3H and B6 mice would respond differently to challenges by dietary calcium depletion and repletion. It has been well documented that dietary calcium depletion causes hypocalcemia, which leads to secondary hyperparathyroidism, subsequently resulting in increased bone resorption. Conversely, dietary calcium repletion reversed the effects, leading to a rapid reduction in bone resorption rate and an increase in bone formation. Changes consistent with this picture were found in both strains of mice.

1. Dietary calcium depletion and repletion. The current studies were intended to compare the skeletal responses of C3H and B6 mice to 2 weeks of dietary calcium (Ca)-depletion, followed by 2 weeks of Ca-repletion. Our initial studies showed that femur dry weight decreased during Ca-depletion in both C3H and B6 mice (by 25% and 19%, respectively, $p<0.001$) and most of this loss was recovered during Ca-repletion (Table 11). Serum alkaline phosphatase (ALP) activity increased during Ca-depletion, in both strains of mice ($p<0.001$), and returned to normal after Ca-repletion (Table 12). Histological analyses of ground cross-sections prepared at the tibio-fibular junction showed that Ca-depletion increased medullary area in both C3H and B6 mice (indicating endosteal bone loss, $p<0.01$), with reversal during Ca-repletion (Table 13). There were no effects of Ca-depletion or repletion on periosteal bone growth. Endosteal bone forming surface and the endosteal mineral apposition rate decreased during Ca-depletion and increased during repletion in both C3H and B6 mice ($p<0.05$). Net bone formation decreased during Ca-depletion in C3H, but not B6, mice ($p<0.01$) and was normal during Ca-repletion, in both strains (Table 14). Endosteal bone resorbing surface and net bone resorption increased during Ca-depletion and decreased during repletion in both strains ($p<0.01$). A supplemental study (of Ca-depletion without repletion) confirmed the effects of Ca-depletion on femoral dry weight and serum ALP activity ($p<0.001$ for each) and showed that Ca-deficiency increased serum PTH ($p<0.05$) and decreased (tibial) cortical bone area and cortical mineral content ($p<0.05$ to $p<0.001$) in both strains of mice (Table 15). Finally, our studies showed that Ca-depletion decreased the number of osteoclast-progenitor cells in the femoral marrow of 4-6 week old C3H and B6 mice ($p<0.02$ for each) (Table 16). Together, these data demonstrate that the skeletal responses to Ca-depletion and repletion are, qualitatively, similar in C3H and B6 mice.

Table 11. Effects of calcium depletion/repletion on femoral dry weight (mg).

Mouse Strain	Time	Control	Treated	Significance
B6	2 Weeks	30.4±0.2	24.8±0.5	p<0.001
B6	4 Weeks	35.6±0.6	33.7±0.5	N.S.
C3H	2 Weeks	30.5±1.0	22.9±1.1	p<0.001
C3H	4 Weeks	37.0±2.8	34.1±0.8	N.S.

Control mice received the normal Ca diet (0.6% Ca) for 2 weeks or 4 weeks and treated mice either received the low-Ca diet (0.01% Ca) for 2 weeks or the low-Ca diet for 2 weeks followed by the normal Ca diet for 4 weeks. Data shown as mean +/- SEM (n=8-10). At the beginning of the study period (i.e., the 0 week basal controls), femur dry weight averaged 21.65 +/- 0.78 and 19.41 +/- 0.54 mg for B6 and C3H mice, respectively. Significance indicates effect of treatment (i.e., effects of Ca-depletion and repletion, as determined by ANOVA). N.S. indicates no significance ($p>0.05$). Two-way ANOVA indicates an effect of treatment ($p<0.001$), but not mouse strain, on bone dry weight.

Table 12. Effect of calcium depletion/repletion on ALP activity.

	B6 Mice	B6 Mice	C3H Mice	C3H Mice
Treatment Group	Serum ALP	Bone Extract ALP	Serum ALP	Bone Extract ALP
Baseline Control	59.6 ± 6.4	2.58 ± 0.16	83.7 ± 7.5[a]	2.45 ± 0.11
2 Week Control	29.9 ± 1.8	2.10 ± 0.17	32.7 ± 1.5	2.11 ± 0.08
Ca-Depletion	48.4 ± 3.3[d]	2.01 ± 0.14	40.2 ± 1.6[a,d]	2.83 ± 0.17[a,c]
4 Week Control	24.1 ± 1.2	1.55 ± 0.16	27.9 ± 2.0	1.36 ± 0.08
Ca-Repletion	27.5 ± 1.6	1.23 ± 0.08	28.0 ± 2.3	2.00 ± 0.14[b,c]

All data shown as mean +/- SEM (n=8-10). Serum ALP reported as mU/mL, bone ALP as mU/mg dry weight of femur. [a] Indicates a significant difference, compared to B6 mice, $p<0.05$, [b] $p<0.005$.
[c] Indicates a significant difference, compared to normal Ca-diet control group of the same age and mouse strain (i.e., an effect of treatment), $p<0.05$, [d] $p<0.001$. 2-way ANOVA shows significant effects of mouse strain and treatment on both serum and bone extract ALP activities, $p<0.001$ for each.

Table 13. Effects of calcium depletion/repletion on endosteal mineral apposition rate and medullary area.

		Endosteal MAR	(μm/day)	Medullary Area	(mm^2)
Mouse Strain	Time	Control	Treated	Control	Treated
B6	2 Weeks	1.49 ± 0.10	1.50 ± 0.14	0.433±0.018	0.493±0.008[a]
B6	4 Weeks	1.44 ± 0.11	1.91 ± 0.14[a]	0.420±0.014	0.454±0.021
C3H	2 Weeks	2.69 ± 0.14[c]	3.16 ± 0.20[c]	0.253±0.007[c]	0.378±0.013[b,c]
C3H	4 Weeks	2.73 ± 0.13[c]	3.18 ± 0.12[a,c]	0.231±0.001[c]	0.279±0.012[a,c]

Medullary area and endosteal MAR measured in ground cross-sections of tibiae prepared at the tibio-fibular junction. Control mice received the normal Ca diet (0.6% Ca) for 2 weeks or 4 weeks and treated mice either received the low-Ca diet (0.01% Ca) for 2 weeks or the low-Ca diet for 2 weeks followed by the normal Ca diet for 4 weeks. All data shown as mean +/- SEM (n=8-10). [a] Indicates an effect of treatment (i.e., compared to control mice of same strain at the same time), $p<0.01$; [b] $p<0.001$. [c] Indicates a difference compared to B6 mice (i.e., at the same time and receiving the same treatment), $p<0.001$.

Table 14. Effects of calcium depletion/repletion on endosteal bone resorption and formation

		Net Resorption	(mm^2 x 10^3)	Net Formation	(mm^2 x 10^3)
Mouse Strain	Time	Control	Treated	Control	Treated
B6	2 Weeks	12.3 ± 15.1	68.4 ± 9.5[a]	18.0 ± 1.8	13.5 ± 2.7
B6	4 Weeks	0.4 ± 13.1	31.7 ± 21.5	19.5 ± 1.1	16.8 ± 1.6
C3H	2 Weeks	26.0 ± 5.4	132.0 ± 12.6[b,c]	29.6 ± 2.2[c]	10.6 ± 2.6[b]
C3H	4 Weeks	4.6 ± 5.5	57.2 11.7[b]	30.4 ± 2.1[c]	35.6 ± 1.6[c]

Net endosteal resorption and formation calculated for ground cross-sections of tibiae prepared at the tibio-fibular junction. Control mice received the normal Ca diet (0.6% Ca) for 2 weeks or 4 weeks and treated mice either received the low-Ca diet (0.01% Ca) for 2 weeks or the low-Ca diet for 2 weeks followed by the normal Ca diet for 4 weeks. All data shown as mean +/- SEM (n=8-10). [a] Indicates an effect of treatment, $p<0.01$ (compared to control group of same mouse strain and same time); [b] $p<0.001$. [c] Indicates a difference, compared to B6 mice (same time and treatment), $p<0.01$. Net bone resorption calculated as the difference between the change in medullary area and net bone formation, during the final two week period (i.e., during the depletion or repletion phase for the 2- and 4-week groups, respectively).

Table 15. Effect of calcium deficiency on bone parameters.

Parameter	Mouse	Control	Ca-Deficient	Difference
Cortical Bone Area	B6	0.535 ± 0.009	0.485 ± 0.010	p<0.05
Cortical Mineral Content	B6	0.532 ± 0.009	0.449 ± 0.011	p<0.005
Cortical Bone Area	C3H	0.666 ± 0.015[b]	0.553 ± 0.18[a]	p<0.001
Cortical Mineral Content	C3H	0.746 ± 0.018[b]	0.570 ± 0.023[b]	p<0.001

Tibial bone density (i.e., cortical bone area and mineral content) determined by pQCT at the tibio-fibular junction. Control mice received the normal Ca diet (0.6% Ca) for 2 weeks and treated mice received the low-Ca diet (0.01% Ca) for 2 weeks. Data shown as mean +/- SEM (n=12/group). Difference refers to the comparison of control vs. Ca-deficient mice, with respect to each parameter (i.e., effects of treatment). [a] Indicates a difference compared to B6 mice of same age and treatment, $p<0.005$, [b] $p<0.001$. Two-way ANOVA shows effects of mouse strain and treatment on both cortical area and cortical mineral content, $p<0.001$ for each.

Table 16. Effects of calcium depletion/repletion on the number of osteoclast progenitor cells in the femoral marrow of C3H and B6 mice.

Study	Mouse Strain	Time	Control	Treated	Difference
1st Study	B6	2 Weeks	322 ± 17	424 ± 10	p<0.02
1st Study	B6	4 Weeks	701 ± 29	808 ± 30	p<0.02
1st Study	C3H	2 Weeks	268 ± 20	164 ± 17	p<0.05
1st Study	C3H	4 Weeks	675 ± 46	723 ± 30	- - - -
2nd Study	B6	2 Weeks	893 ± 35	716 ± 49[a]	p<0.02
2nd Study	C3H	2 Weeks	474 ± 19	371 ± 31[a]	p<0.02

Osteoclast progenitor cells were identified in replicate cultures (n=6-9) of pooled femoral marrow cells. In the first study, control mice received the normal Ca diet (0.6% Ca) for 2 weeks or 4 weeks and treated mice either received the low-Ca diet (0.01% Ca) for 2 weeks or the low-Ca diet for 2 weeks followed by the normal Ca diet for 4 weeks. In the second study, control mice received the normal Ca diet and treated mice received the low-Ca diet, for 2 weeks. Data shown as mean +/- SEM. Mice in the first study were 7 and 9 weeks of age after 2 weeks and 4 weeks of treatment. Mice in the second study were 6 weeks of age at the end of the 2 week protocol. Difference refers to the comparison of control vs. treated groups at each time point. [a] Indicates a difference compared to B6 mice of same

age and treatment group, $p<0.001$. Two-way ANOVA shows significant effects of mouse strain and treatment, $p<0.001$ for each.

III. Research Aims for year 3. Our original goals for year 3 of this project was a) to continue processing bone samples from the 14 BXH RI strains plus companion B6 controls as well as any remaining B6xC3 F1 hybrid bone specimens for histomorphometric analyses of bone formation and resorption processes; b) to continue evaluate serum samples of these mouse strains and their progeny for biochemical markers for bone formation and resorption; and c) to analyze serum or bone samples of any additional groups of mice obtained from the Jackson Laboratory colonies.

However, on the basis of our progress thus far, we have revised our proposed research goals for year 3 as the following: a) we will complete any bone histomorphometric measurements that have not been finished during the first two years; b) we will focus on vertebra samples and also samples obtained from F1 progenies; c) because our recent data indicate that congenic mice are probably more suitable for genetic analyses than BXH RI strain, we will shift our emphasis to the congenic mice produced in the Jackson Laboratory; d) we will also continue to evaluate the possibility that the C3H mice and B6 mice may differ in these responsiveness to bone formation and resorption stimuli.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 27 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the enclosed list of technical documents. Request the limited distribution statement assigned to the documents listed be changed to "Approved for public release; distribution unlimited." These documents should be released to the National Technical Information Service.

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FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
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